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Original Article

# Analysis of the azoreductase gene harbored by *Alcaligenes* sp. YB4 capable of concurrent removal of sulphonated azo dye and hexavalent chromium

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## Abstract

Continuous discharge of textile wastewater consisting of variety of pollutants is a serious threat to ecosystems. Microbial bioremediation might serve as an effective approach for treating these unwanted contaminants. In this study, several bacteria isolated from textile wastewater were studied for decolorization of Congo red (CR) dye. The strain *Alcaligenes* sp. YB4 showed the most efficient potential to decolorize CR dye. Moreover, this strain efficiently decolorized CR while concurrently removing hexavalent chromium [Cr(VI)] in the same medium with maximum removal (> 90 %) of both pollutants at pH 7 and pH 8. The potential of YB4 for concurrent removal of both pollutants was observed to decrease with increasing concentration of NaCl. Similarly, *Alcaligenes* sp. YB4 efficiently removed the 91.6 % of CR and 95.7 % of Cr(VI) simultaneously, under static condition as compared to the shaking condition. While MS media amended with yeast extract showed about 92.2 % and 90.1 % removal of CR and Cr (VI) within 48 hours of incubation, respectively. Moreover, it was also noticed that presence of heavy metals effected the concurrent removal of both pollutants. The in-silico analysis of the azoreductase amplified from the strain YB4 identified the binding of CR with azoreductase and proposed the hypothesis that their association may be the primary cause of CR degradation. This study indicated that *Alcaligenes* sp. YB4, having azoreductase gene, is a potential resource to treat textile wastewater.

**Keywords**: Congo red, Hexavalent chromium, Azoreductase, Molecular docking, Azo dyes

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## Introduction

The economy of Pakistan mainly depends on revenue generated by the textile industry. Around 25% of the industrial value-addition is contributed by this sector, and it employs approximately 40% of the industrial labor force. Textile products have consistently maintained an average share of approximately 61.24% in national exports, except for seasonal and cyclical fluctuations (Pakistan Economic Survey,

2021-22). However, on the other hand, textile industry is utilizing a significant amount of water, 0which can pose severe risks to groundwater quality due to a substantial number of recalcitrant pollutants in untreated wastewater discharged into the mainstream (Angelis-Dimakis et al., 2016; Li et al., 2019). As a result, it is considered as second after the agricultural industry for polluting the water resources (Kant, 2012). Additionally, Noreen et al. (2017) have also highlighted the discharge of untreated wastewater by the textile industry as a significant concern. The wastewater of this industry mainly contains azo dyes, heavy metals, and different salts (Saratale et al., 2009b; Velusamy et al., 2021). Untreated wastewater with a high pollutant load is a serious concern for all water lives, humans, and agricultural lands as well (Imran et al., 2015a, 2015c; Daud et al., 2017).

Azo dyes have azo bond (−N=N−) in their structure while occupying a big space in all dye forms, as they constitute almost 50% of all dyes with a total of 700,000 tons per annum worldwide (Anwar et al., 2014; Elgarahy et al., 2019). Their economic value, stability to absorb photons, variety of colors, and power to resist oxidizing products make them an utmost need of several industries, including textile, pharmaceutical, and printing etc. (Dawkar et al., 2010; Koop and van Leeuwen, 2017; Bashir et al., 2020; Alaskar and Hassabo, 2021). During textile processing, 10–15 percent of the dye fails to approach the binding sites of fibers and discharged into the environment (Saratale et al., 2009a; Chaudhary et al., 2013; Singh et al., 2015; Sarkar et al., 2017; Tara et al., 2019). The presence of azo dyes disturbs water quality in many ways such as reduction of water transparency as well as aesthetic value and limited oxygen supply as well as light penetration in the water (Hussain et al., 2013; Imran et al., 2015b; Bhatia et al., 2018; Aleem et al., 2020). Many environmental problems are linked to this serious issue of toxic pollutants (Ayed et al., 2010; Qu et al., 2012; Rawat et al., 2016). Azo dyes produce some intermediate products that results into cancerous cells in living bodies (Khehra et al., 2006; Elbanna et al., 2010; Yusuf et al., 2017; Alshehrei, 2020). The germination rate of plants is affected by azo dyes and many of the plant species face biomass reduction as well (Ghodake et al., 2009; Kumar et al., 2023). Many of the water parameters (COD, BOD) are affected by azo dyes (Saratale et al., 2009a; Imran et al., 2015b; Maqbool et al., 2016; Reck et al., 2018).

In the textile wastewater, in addition to dyes, a significant quantity of metals and salts are also present in it (Ngah and Hanafiah, 2008; Imran et al., 2015a; Ismail and Sakai, 2022). Metal salts are used in the industry as chemicals used in dying process, metal complexed azo dyes, and salts heaving different metals (Tuzen et al, 2008; Imtiazuddin et al., 2014; Singha et al., 2021). Chromium is often used for better fixation of dyes on textile products (Anwar et al., 2014; Velusamy et al., 2021). Among various chromium forms, hexavalent chromium [Cr(VI)] is often more toxic to many plants, animals, and microorganism (Mahmood et al., 2013; Anwar et al., 2014; Nakkeeran et al., 2018; Rahman and Thomas, 2021).

So, based on the facts gathered by scientific literature, it is an established fact that azo dye and Cr(VI) are potential threats to the environment. This situation demands sustainable approaches for their removal from soil and water resources. Biodegradation is often considered as an environment friendly approach for treatment of wastewater (Anwar et al. 2014; Maqbool et al., 2015; Imran et al., 2015a; Rathi and Kumar, 2022). Numerous researchers have described the microbial degradation of azo dyes (Imran et al., 2014; Najme et al., 2015; Bilal et al., 2022). Similarly, reports are also present which indicate that that microbes have the ability to detoxify the Cr(VI) (Maqbool et al., 2015; Rahman and Thomas, 2021; Shan et al., 2023; Abdulmalik et al., 2023; Rubab et al., 2023; Liaqat et al., 2023). Besides, few researchers also successfully isolated such microbes which can reduce the azo dyes and Cr(VI) simultaneously (Hussain et al., 2017; Maqbool et al., 2018; Hussain et al., 2020). However, isolation of novel more efficient microbial strains having the potential for bioremediation of both of these pollutants from the wastewater is needed in this regard.

Faisalabad is one of the industrial cities of Pakistan with a wide capacity of the textile sector with various processing units. These industries produce a vast amount of wastewater loaded with toxic pollutants including azo dyes and Cr(VI) that are discharged into different drains such as Paharang and Madhuana drains with no or little treatment that ultimately falls into Ravi and Chenab rivers (Azizullah et al., 2011; Imtiazuddin et al., 2012; Mohib et al., 2021; Sohail et al., 2022). Hence, novel approaches for bioremediation of such wastewater should be devised by isolating novel and more efficient bioresources which can simultaneously reduce the azo dye and Cr(VI). In this context, this study was designed for

finding a novel bacterial strain possessing the potential for remediation of dyes and Cr(VI) simultaneously in the provided media. Moreover, the capabilities of the strain were tested under varying cultural as well as incubation conditions and the azoreductase enzyme was amplified, sequenced and analyzed using bioinformatics tools.

## Material and Methods

### Collection of textile wastewater samples

Textile wastewater samples were taken in pre-sterilized reagent bottles from different sites of the Faisalabad (Location-1: 31.471° North & 73.061° East and Location-2: 31.467° North & 73.051° East) nearby the textile industrial units. After measuring the EC and pH of fresh wastewater, these wastewater containing bottles were preserved in refrigerator at 4 °C to prevent any change in the physiochemical and biological properties until its use.

### Chemicals and media

Dyes i.e., reactive black-5 (RB5), reactive red-2 (RR2), reactive yellow-2 (RY2), congo red (CR), ramazole brilliant blue R (RBBR) and orange II sodium salt (OII), used in this study, were purchased from Sigma-Aldrich. Mineral salt media (MSM) composed of $Na_2HPO_4$ (1.0 g $L^{-1}$), $KH_2PO_4$ (1.0 g $L^{-1}$), $MgSO_4.7H_2O$ (0.5 g $L^{-1}$), NaCl (1.0 g $L^{-1}$), $CaCl_2$ (0.1 g $L^{-1}$), Dye (200 mg $L^{-1}$), Yeast Extract (4 $gL^{-1}$), and Agar Powder (15 $gL^{-1}$ for solid media) was used to isolate the bacteria. When the pH of the media was required to maintain, 6M HCl & 10M NaOH were used.

### Isolation, screening and identification of dye decolorizing bacteria

Decolorizing bacteria were isolated from textile wastewater was done by following the enrichment technique. For this purpose, 10 mL of each wastewater was taken in separate presterilized 250 mL volume conical flasks and 90 mL of dye (200 $mgL^{-1}$) amended MSM, was mixed in each flask. These flasks were tightly sealed and then put under shaking condition in incubator for 24h at 30 °C. There was an additional flask which had dye amended MSM but there was no wastewater added in it. It was used as control. Samples were collected, centrifuged (10000 rpm for 10 min) and evaluated by using UV-visible spectrophotometer at $\lambda_{max}$. Decolorization % of each sample was determined by using the formula;

$$Decolorization\ (\%) = \frac{control - sample}{control} \times 100$$

When more than 50% decolorization was observed in flasks, then 10ml was shifted to another set of flasks having the 90mL of freshly prepared MSM amended with 200 mg $L^{-1}$ dye and incubated these flasks under same conditions as mentioned above. After 5 cycles, 100µL from each culture was used for serial dilution ($10^{-1}$ – $10^{-6}$) and 100 µL from each dilution were spread on the MSM plates and placed them in incubator at 30 °C. After 48 h of incubation, different colonies appeared on these plates.

For screening, 100 selected colonies were separately resuspended in MSM amended with azo dyes and monitored their growths. Eighteen milliliters of fresh MSM amended with azo dye was taken and two milliliters of each colony growth in MS medium were suspended in separate test tubes and incubated under similar conditions. After 24h and 48h, aliquots (1.5 mL) were collected, centrifuge at 10000 rpm for 10 min and used for evaluation of decolorization percentage by the procedure described above. Among these strains, YB4 was found most efficiently decolorizing bacterium. So, the strain YB4 was used for further characterization by providing different environmental conditions. The strain YB4 was identified through amplification and analyses of its 16S rDNA gene following the protocols and programs already described by Hussain et al. (2011).

### Efficiency of *Alcaligenes* sp. YB4 for decolorization of diverse dyes

The efficiency of the strain YB4 for decolorization of different structured dyes was estimated in MS media. 18 mL of MS media (200 $mgL^{-1}$ of each dye separately) was added with 2 mL culture of YB4 while maintaining the bacterial density ($OD_{600}$) of 0.05 and incubated in a static incubator at 30 °C. Experiment was conducted in triplicate for each dye with uninoculated controls. The samples were collected from each culture at different regular time intervals and then centrifuged at (10000 rpm for 10 min). The supernatants were analyzed spectrophotometrically at $\lambda_{max}$ of each dye (RB5-597nm, RR2-540nm, RY2-404nm, CR-497nm, RBBR-591nm, OII-486nm). Decolorization (%) was estimated by using the equation described in the above section by comparing with the un-inoculated control for each dye.

**Removal of Cr(VI) at its varying initial concentrations by *Alcaligenes* sp. YB4**

The potential of YB4 to remove the Cr(VI) at its varying initial concentrations (5, 10, 20, 50 mg/L) was estimated in MSM. 18mL of fresh MSM with different concentrations of Cr(VI) was added with 2 mL of YB4 culture while maintaining the bacterial density ($OD_{600}$) of 0.05. The required concentrations of Cr(VI) were obtained by using the potassium dichromate ($K_2Cr_2O_7$). The triplicates tightly closed glass tubes containing the cultures were kept in a static incubator at 30 °C along with controls (un-inoculated). Samples were taken from each test tube after regular intervals (24 h and 48 h), centrifuged at 6000rpm for 10 mins, and the supernatant was used for the determination of Cr(VI) as already reported by Anwar et al. (2014).

**Concurrent removal of CR and Cr(VI) by *Alcaligenes* sp. YB4 at different pH**

The effect of pH was estimated on the activity of strain YB4 and optimum pH was determined. Fresh MSM added with CR (@200 mg $L^{-1}$) and Cr(VI) (@10 mg $L^{-1}$) was prepared and varying pH values (5, 6, 7, 8, 9, 10) were separately maintained by using HCl and NaOH solutions. Whole experiment was run under same conditions as mentioned above. Sampling was done at regular intervals and then % decolorization and Cr(VI) reduction were determined by using protocol as discussed before.

**Concurrent removal of CR and Cr(VI) by *Alcaligenes* sp. YB4 at different levels of salt**

For this purpose, fresh MSM added with CR (@200 mg $L^{-1}$) and Cr(VI) (@10 mg $L^{-1}$) was prepared. Different concentrations of NaCl (0, 5, 10, 20, 50, 100 g/L) were separately developed in media. 18 mL media with each concentration of NaCl was poured in glass test tube with cap and 2 mL of bacterial culture was added in those test tubes. Triplicates of tubes were kept in a static incubator at 30 °C along with control (un-inoculated). Samples were taken after regular intervals and examined for CR decolorization (%) and Cr(VI) reduction by following the methods as mentioned above.

**Concurrent removal of CR and Cr(VI) by *Alcaligenes* sp. YB4 in static and shaking conditions**

The efficiency of simultaneous CR decolorization and Cr(VI) reduction of YB4 at static and shaking incubation conditions was estimated. Three replicates of two sets of fresh MS media having CR (@200 mg $L^{-1}$) and Cr(VI) (@10 mg $L^{-1}$), and inoculated with YB4 in tightly sealed test tubes were incubated in static incubator and shaking incubator (150 rpm) set at 30 °C separately. The samples were collected at regular intervals and analyzed by the protocols as mentioned above.

**Impact of various C sources on immediate removal of CR and Cr(VI) by *Alcaligenes* sp. YB4**

The efficiency of YB4 to decolorize CR and reduce Cr(VI) simultaneously in the presence of various C sources i.e. yeast extract, maltose, D-mannitol and glucose was also tested in MSM. For this purpose, fresh MSM containing 200 $mgL^{-1}$ CR and 10 $mgL^{-1}$ Cr(VI) supplemented with 4 $gL^{-1}$ of each C source filled in tightly sealed test tubes. The cultures were incubated in same conditions and immediate removal of CR and Cr(VI) was analyzed by same protocols as mentioned above.

**Minimum Inhibitory concentration (MIC) of metal ions for YB4**

Minimum concentration of different heavy metals (Pb, Cd, Ni, Co, Zn, Cr) was measured against the strain *Alcaligenes* sp. YB4. MIC of each heavy metal was measured separately using the repeated streaking method in Nutrient Agar plates. YB4 was spread over nutrient agar plates amended with different concentrations of heavy metal ranging from 10 to 2000 $mgL^{-1}$ of each heavy metal. The values of MIC were recorded by examining the growth of YB4 in each plate under the digital colony counter.

**Impact of heavy metals on immediate removal of Congo red and Cr(VI) by YB4**

The potential of YB4 to remove CR and Cr(VI) simultaneously was also evaluated in MS medium containing the heavy metals (Cd, Ni, Co, Zn, Pb). For this purpose, freshly prepared MSM containing 200 $mgL^{-1}$ CR and 10 mg/L Cr(VI) along with varying concentrations of mixture of heavy metals (0%, 1%, 2%, 5%, 10%, 20% of MIC) were inoculated separately with YB4. 18mL of this amended MSM was poured in glass test tubes and 2mL of liquid culture of YB4. The tubes were tightly sealed and incubated under static conditions at 30 °C. Sampling and measurement for dye decolorization and Cr(VI) reduction were carried out as already described above.

**Amplification of azoreductase gene**

The amplification of azoreductase gene was done from the crude DNA of the strain YB4 using AZR1f and AZR1r primers by using the PCR master-mixture and program already described by Mahmood et al. (2017). 2.5µL of crude DNA in total reaction mixture of 25µL with setting the thermocycler conditions for 30 cycles, consisting of 1 minute at 95°C, 30 seconds at 55°C, and 1 minute at 72°C. The initial denaturation and final extension steps were carried out at 95°C for 5 minutes and 72°C for 10 minutes, respectively. The amplified azoreductase product was finally sequenced from Macrogen, Korea.

**Bioinformatics analysis of amplified and sequenced azoreductase gene**

The amplified and sequenced azoreductase gene was translated by using the ExPASY translate tool (https://www.expasy.org/resources/translate). The translated protein sequences were compared with other protein sequences by using BLASTp tool against the protein database bank (pdb). 3D protein structure of YB4 deduced azoreductase protein was predicted by using the i-TASSER tool. To ensure the physical and chemical properties of the achieved azoreductase protein sequences, physicochemical characterization of the sequences was performed by using the ExPASY-ProtParam tool. Different parameters like number of amino acids, molecular weight, theoretical isoelectric point (pI), grand average of hydropathicity (GRAVY), molecular weight, aliphatic index and instability index was calculated by using this tool.

A docking study between the predicted protein structure and the CR structure was carried out by using the PatchDock (https://bioinfo3d.cs.tau.ac.il/PatchDock/). Further, the best match was refined by using the FireDock server. PatchDock calculates the docking transformation between two molecules which identified the ligand position in the receptor with maximum covered area and minimum steric hindrance. It also calculates the global energy (glob), softened attractive and repulsive van der Waals energy (aVdW, rVdW), atomic contact energy (ACE) and insideness measure. After molecular docking analysis, best docked structures were subjected to evaluate the molecular interactions between CR dye and azoreductase protein using UCSF Chimera.

## Results

**Isolation and identification of YB4**

Among the tested isolates, YB4 showed the most efficient potential of CR decolorization under set conditions. The analyses of the sequence of 16S rDNA amplified from YB4 showed maximum similarity (99%) with the several *Alcaligenes* species. On the other hand, the phylogenetic tree was constructed using the neighbor-joining method with the 16S rRNA sequences of YB4 and several other strains retrieved from the GenBank database. In phylogenetic tree, the strain YB4 clustered itself with the genus *Alcaligenes* (Figure 1). After conducting comparative and phylogenetic analyses of isolate YB4 in relation to other bacterial strains belonging to the genus *Alcaligenes*, the bacterial isolate YB4 was designated as *Alcaligenes* sp. YB4.

**Decolorization of various dyes by YB4**

The YB4 strain efficiently but variably decolorized different azo dyes (Table 1). After 24 h, the maximum decolorization by *Alcaligenes* sp. YB4 observed in CR which is 60.87% followed by 50.26% and 42.22% decolorization for RR2 and RB5, respectively. After 48 h, the maximum decolorization was again observed for CR which is 89.45% followed by RR2 and RB5 which were decolorized up to 87.6% and 78.73%, respectively. However, it showed less efficacy of decolorization of orange-2 sodium, remazol brilliant blue R and RY2 as it decolorized these dyes up to 45.57%, 28.35%, and 13.83%, respectively, after 48 h incubation period. As the maximum decolorization potential was observed in CR dye by the YB4 strain so further experiments were conducted using CR dye.

**Cr(VI) reduction potential of YB4 at different initial concentrations of Cr(VI)**

The data regarding reduction of Cr(VI) by YB4 at its varying initial concentrations indicated that the maximum Cr(VI) reduction (95.5%) was observed in the medium having 10 $mgL^{-1}$ of Cr(VI) followed by at 5 $mgL^{-1}$ which was reduced up to 91.71% after 48 h incubation (Figure 2). As the concentration increases to 20 and 50 $mgL^{-1}$, the reduction potential was also minimized and decrease up to 18.82 and 11.32% of the added concentrations of Cr(VI), respectively, after 48 h incubation.

**Table-1. Dye decolorization of Alcaligenes sp. YB4 against the different structured azo dyes**

| Dyes | Chemical Formula | $\lambda_{max}$ | Decolorization % | |
|---|---|---|---|---|
| | | | 24hrs | 48hrs |
| **Reactive Black 5** | $C_{26}H_{21}N_5Na_4O_{19}S_6$ | 597 | 50.26±1.24 | 78.73±1.11 |
| **Reactive Red 2** | $C_{19}H_{10}Cl_2N_6Na_2O_7S_2$ | 540 | 42.22±0.59 | 87.60±5.61 |
| **Reactive Yellow** | $C_{25}H_{15}C_{l3}N_9Na_3O_{10}S_3$ | 404 | 4.23±3.24 | 13.83±2.37 |
| **Congo Red** | $C_{32}H_{22}N_6Na_2O_6S_2$ | 497 | 60.87±5.44 | 89.45±1.95 |
| **Remazol Brilliant Blue R** | $C_{22}H_{16}N_2Na_2O_{11}S_3$ | 591 | 16.24±1.78 | 28.35±5.22 |
| **Orange II Sodium Salt** | $C_{16}H_{11}N_2NaO_4S$ | 486 | 12.57±3.14 | 45.57±6.07 |

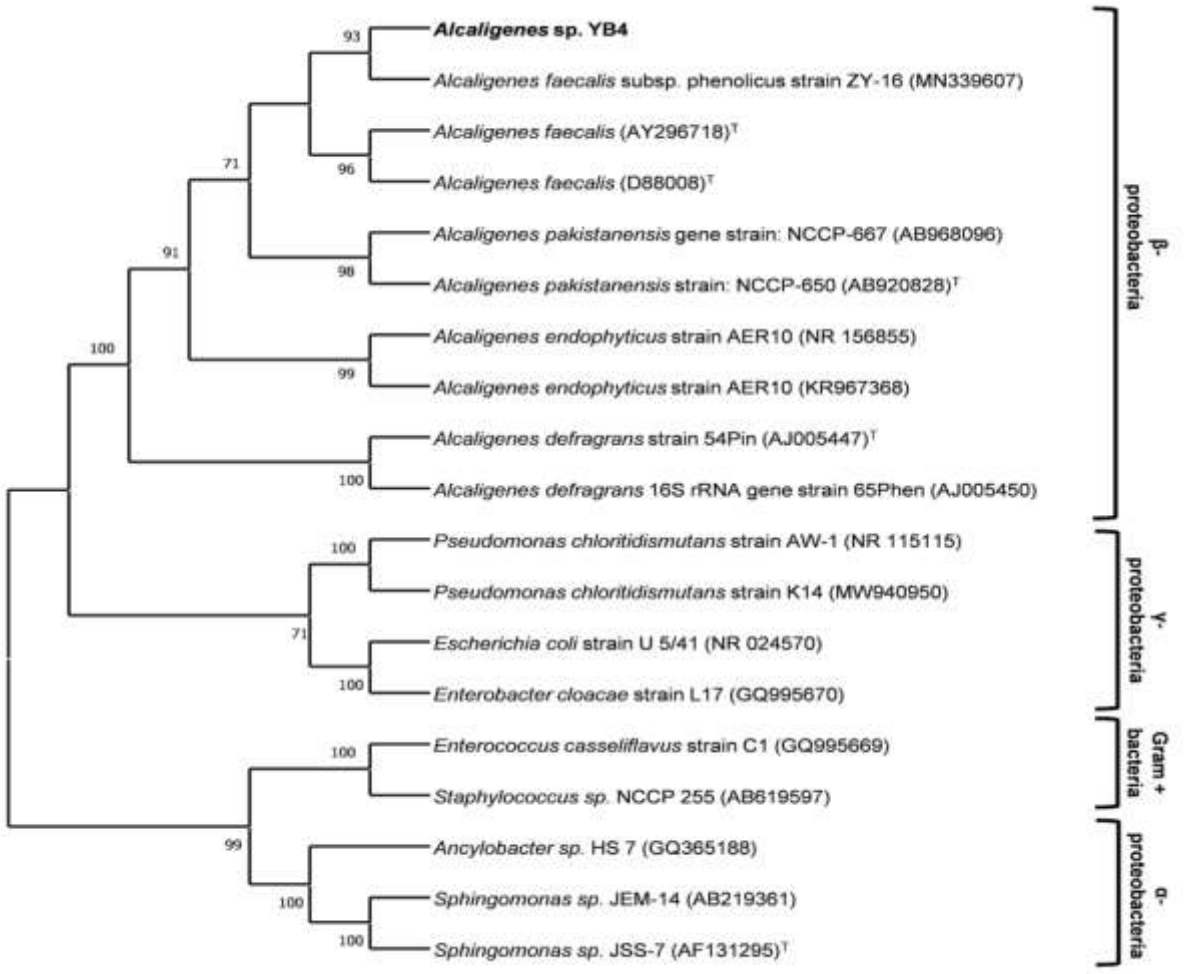


**Figure-1. Phylogenetic tree of YB4 with other strains construct by neighbor joining method**

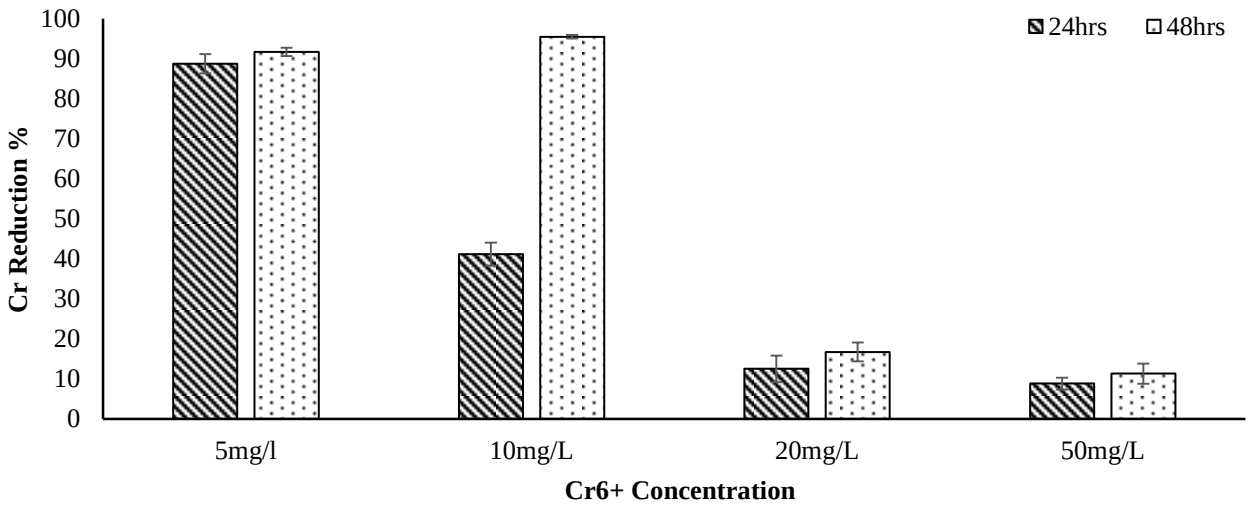


**Figure-2. Cr(VI) reduction by YB4 at different initial concentrations of Cr(VI)**

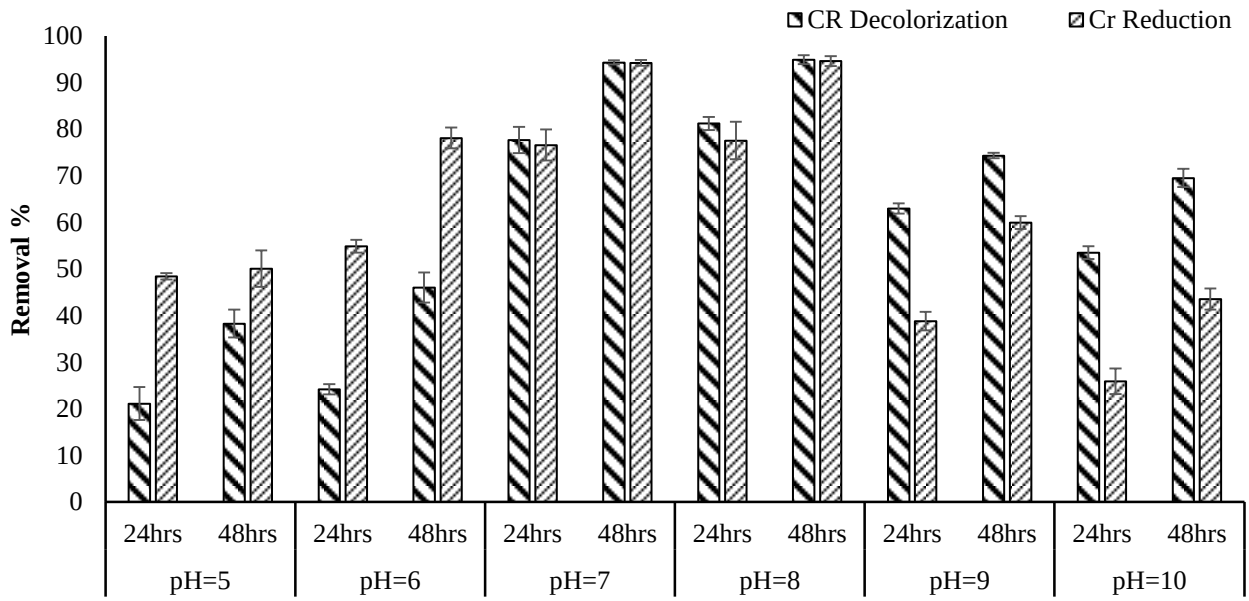


**Figure-3. Simultaneous removal of CR and Cr(VI) at different pH values**

**Simultaneous removal of CR and Cr(VI) by YB4 at different pH values**

While studying the impact of pH on simultaneous CR decolorization and Cr(VI) reduction by the strain YB4, the results showed that almost complete simultaneous removal (>90%) of CR dye as well as Cr(VI) was observed after 48 h incubation at pH 7 and pH 8 (Figure 3). Over 48 h incubation, the strain YB4 carried out 90.7% and 91.2% decolorization of CR at pH 7 and pH 8, respectively. However, at this time, this strain decolorized only 36.5%, 44.2%, 71.4% and 66.8% of CR at pH values of 5, 6, 9 and 10, respectively. Simultaneously, Cr(VI) reduction was also estimated at different pH values. Results indicated that over 48 h incubation period, the maximum Cr(VI) reduction was observed at pH values of 7 and 8 which was recorded as 90.6% and 90.9%, respectively. However, at pH values of 5, 6, 9 and 10, the Cr(VI) reduction values were recorded as 48.2%, 75.1%, 57.6% and 41.9%, respectively, after 48 h incubation period.

**Simultaneous removal of Congo Red and Cr(VI) by YB4 at different levels of NaCl**

The presence of varying salt concentrations affected the simultaneous removal of both CR and Cr(VI) by YB4 (0, 5, 10, 20, 50, 100 $gL^{-1}$) as presented in Figure 4. The results indicated that the CR and Cr(VI) removing potential of YB4 was decrease with the increase of salt concentration. After 48 h incubation, 90% decolorization of CR was observed in the medium without NaCl. Over the same incubation time, with the increase in salt content to 5 and 10 $gL^{-1}$, the decolorization (%) was decreased to 74.7% and 60.3%, respectively. Meanwhile, the strain YB4 decolorized the 25.4%, 18.7% and 12.4% of CR in the media amended with the 20, 50 and 100 g/L NaCl salt, respectively.

Similarly, the reduction of Cr(VI) decreased as the salt concentration increased. Maximum reduction of Cr(VI) (87.4%) was observed in a medium having 0g/L of NaCl salt during a 48-hour incubation period. Over the same incubation period, with the increase in NaCl concentration to 10, 20, 50 and 100 g/L, the Cr(VI) reduction values were observed to be 82.4%, 67.9%, 29.6% and 24.5%, respectively.

**Immediate removal of Congo Red and Cr(VI) by YB4 under static and shaking conditions**

The results showed that *Alcaligenes sp.* YB4 more efficiently removed the CR and Cr(VI) immediately under the static condition as compared to the shaking condition (Figure 5). It was observed that 91.6% decolorization was observed in static conditions after 48h of incubation, while, it was 52.4% under shaking condition at the same incubation time. Similarly, the maximum Cr(VI) reduction (95.7%) was also observed under static condition. During the period of incubation for 48 hours, 51.4% of the initially added Cr(VI) was removed under shaking condition.

**Simultaneous removal of Congo red and Cr(VI) by YB4 by using different carbon sources**

While studying the effect of different C sources on the simultaneous removal of CR and Cr(VI), The findings indicated that the maximum simultaneous removal of CR and Cr(VI) occurred in the MSM that had been supplemented with yeast extract (Figure 6). The decolorization percentage was recorded as 92.2% while Cr(VI) reduction was 90.1% in the yeast extract amended media after the 48h incubation. The maximum CR decolorization, after the yeast extract, was observed in the media amended with D-mannitol (55.4%) followed by maltose (48.3 %) and glucose (37.6%). Similarly, the maximum Cr(VI) reduction, after the yeast extract, was also observed in the media amended with D-mannitol (75.7%) followed by maltose (42.7%) and glucose (35.1%) (Figure 6).

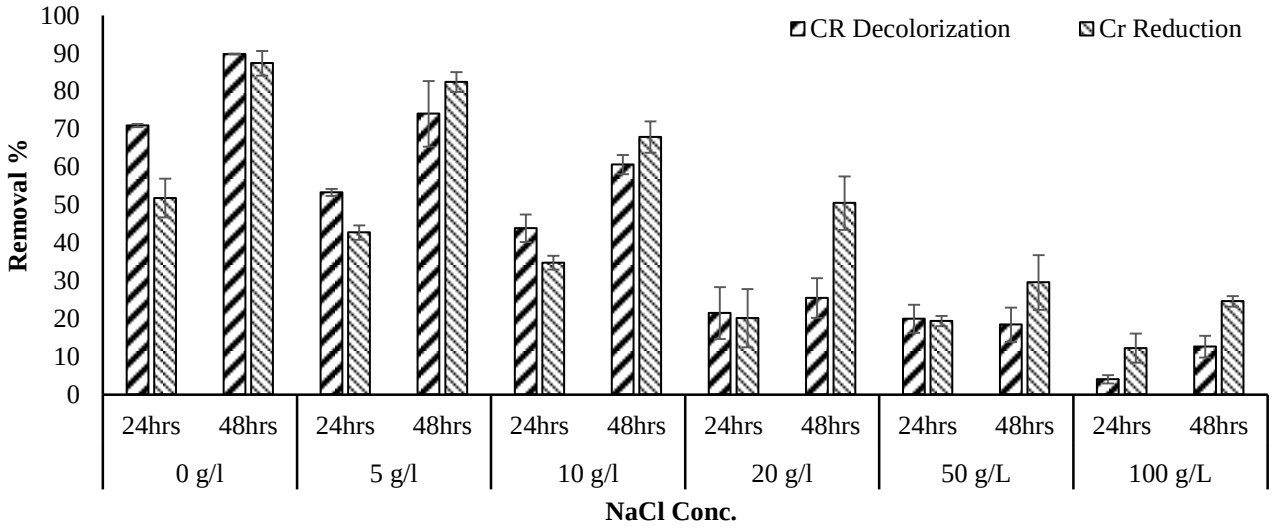


**Figure-4. Simultaneous removal of CR and Cr(VI) by *Alcaligenes* sp. YB4 in the presence of NaCl**

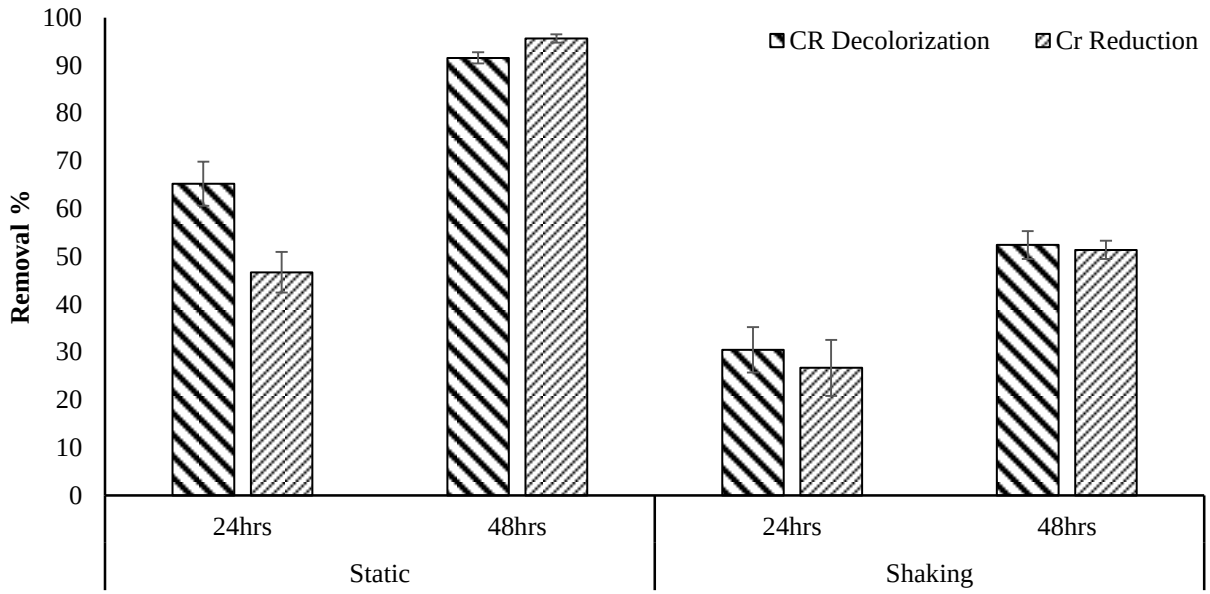


**Figure-5. Simultaneous removal of CR and Cr(VI) by *Alcaligenes* sp. YB4 under static and shaking conditions**

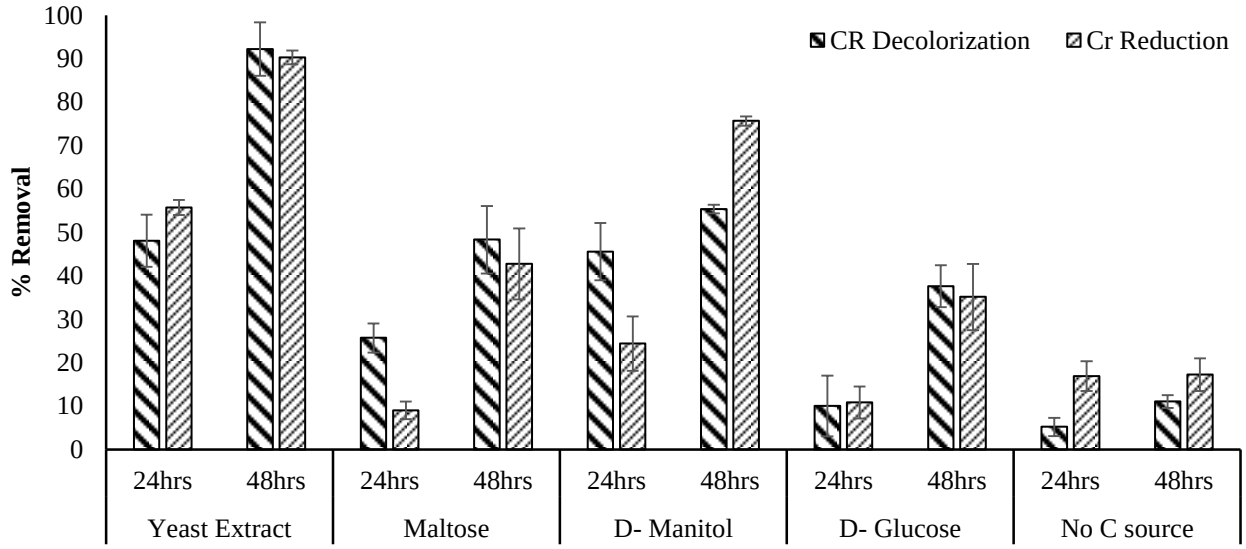


**Figure-6. Simultaneous removal of CR and Cr(VI) by YB4 by using different carbon sources**

**Table 2. Minimum inhibitory concentrations of various metals for YB4**

| Heavy Metals | Minimum Inhibitory Concentration (mM) |
|---|---|
| Lead | 2.41 |
| Cadmium | 1.78 |
| Nickel | 1.70 |
| Cobalt | 8.48 |
| Zinc | 9.17 |
| Chromium | 19.23 |

**MIC of various metals against *Alcaligenes sp.* YB4**

The results showed that the strain YB4 exhibited a dissimilarity in resisting the presence of metal ions (Table 2). The MIC values of $Cr^{6+}$, $Co^{2+}$, $Zn^{2+}$, $Pb^{2+}$, $Cd^{2+}$and $Ni^{2+}$ against the strain YB4 were observed to be 19.23, 8.48, 3.06, 2.41, 1.78 and 1.70 mM, respectively (Table 2).

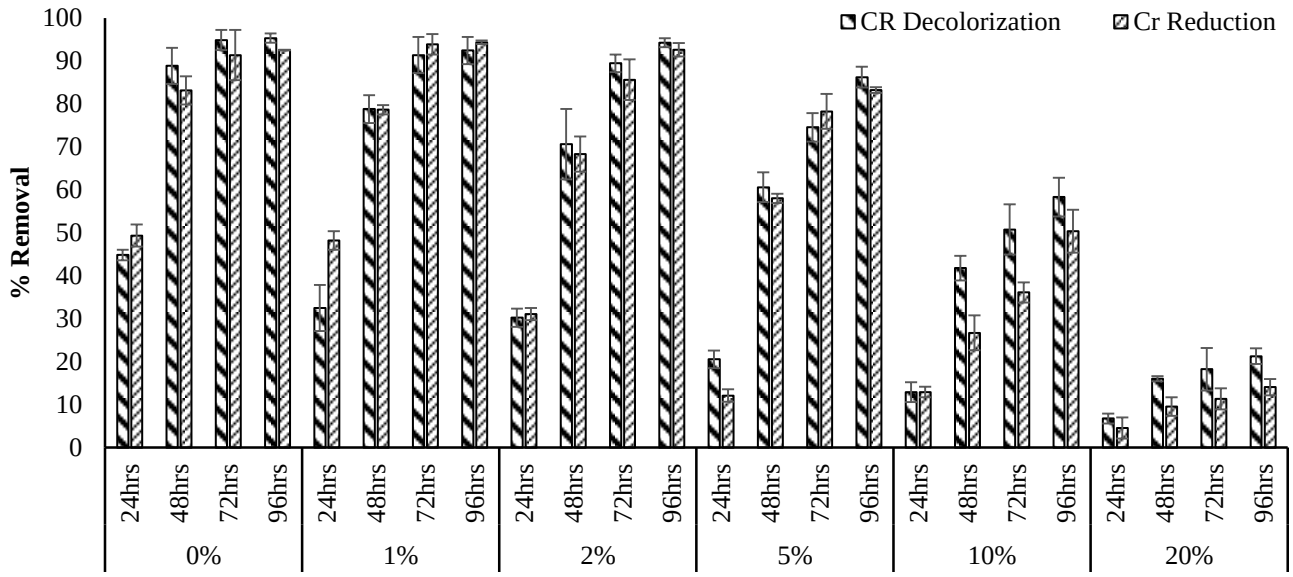


**Figure-7. Simultaneous removal of CR and $Cr^{6+}$ in the media containing metal ions**

**Effect of heavy metals on Congo red decolorization and Cr(VI) reduction by *Alcaligenes* sp. YB4**

The findings indicated that the presence of heavy metals in MSM had an impact on the efficiency of strain YB4 in the removal of CR and Cr(VI) and the efficacy of strain was decreased with the increase in the concentrations of heavy metals (Figure 7). The maximum decolorization of CR (88.9%) was observed in the medium without the mixture of heavy metals after 72 h incubation. At 0, 1, 2 % of heavy metal mixtures, the CR and Cr(VI) were observed to be almost completely removed (>90%) over 72 h incubation. However, at 5, 10, 20 % of heavy metals mixtures, the decolorization percentage was decreased and the maximum decolorization was observed at 5% concentration of heavy metals (86.2%) followed by the heavy metals' concentration of 10% (58.3%) and 20% (21.2%).

Similarly, the reduction of Cr(VI) was decreased as the concentration of heavy metals increased, with the highest reduction of Cr(VI) occurring in the absence of heavy metals. >90% reduction was observed in 1 and 2% of heavy metals' concentrations. But at 5, 10 and 20% of MIC, 83.25, 50.37 and 14.09% Cr(VI) reduction was observed, respectively.

**Bioinformatic analysis of azoreductase product**

173 amino acids were found after the translation of nucleotide sequence of YB4 azoreductase gene. The deduced protein, after the comparative bioinformatic analysis, was found as NADH-ubiquinone: oxidoreductase which is responsible for the decolorization of azo dyes in the YB4. While the BLASTp analysis showed the near most resemblance of protein with the FMN-dependent NADH-azoreductase. This analysis predicted that the YB4 protein has NADH-ubiquinone: oxidoreductase activity, while after the comparison of YB4 protein sequence with protein database bank (pdb), it showed resemblance with the NADH-azoreductase activity. 3D protein structure was predicted by using the i-Tasser online tool. Further, the protein structure was validated with the Ramachandran plot which showed the maximum amino acids in the allowed region and fewer amino acids in the disallowed region. The results indicated that 89.9% of total amino acids of predicted protein were fall in most favorable region while only 1.4% of total amino acids were fall in the disallowed region. Further, the Ramachandran plot also showed the total number of residues which were 174, Glycine (Gly) & Proline (Pro) residues were 17 and 6 respectively, and the end residues excluding the Gly and Pro were 3.

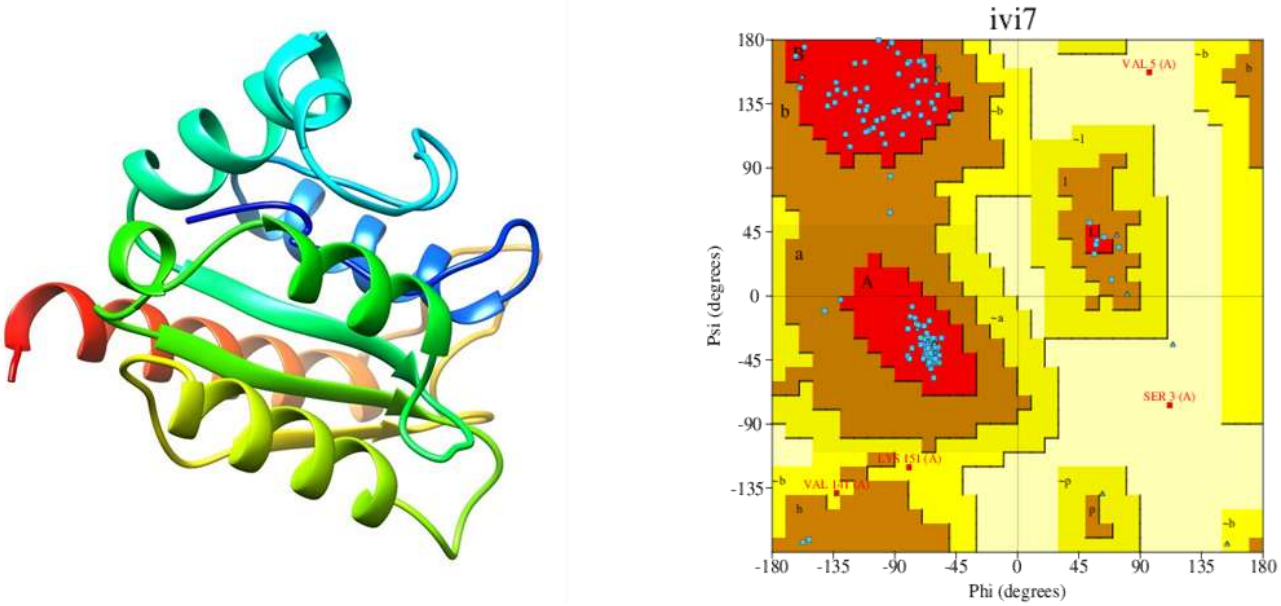


**Figure-8. 3D structure of azoreductase enzyme from YB4 and its validation with Ramachandran plot**

Protein Ligand Docking studies was done on the PatchDock online tool where the docking was performed between the azoreductase enzyme as a protein and CR dye performed as ligand. Then, it measured the active residues of the protein. So, the results showed that ARG (11.A), PHR (13.A), GLY (14.A), ARG (15.A), GLU (73.A), TYR (74.A), HIS (75.A), ASN (76.A), GLY (138.A), VAL (141.A), GLN (142.A) and ASP (143.A) are the active sited where the ligand attach on the targeted protein. This docking system also mentioned the global energy, attractive and repulsive van der Waals energy, atomic contact energy and insideness measure which are -38.13 Kcal/mol, -25.17 & 10.66 Kcal/mol, -09.19 Kcal/mol, 3.30 respectively. As the active sites of azoreductase where the CR dye was attached, it can be hypothesized that this attachment of ligand on protein is a key factor for the CR decolorization in the presence of azoreductase

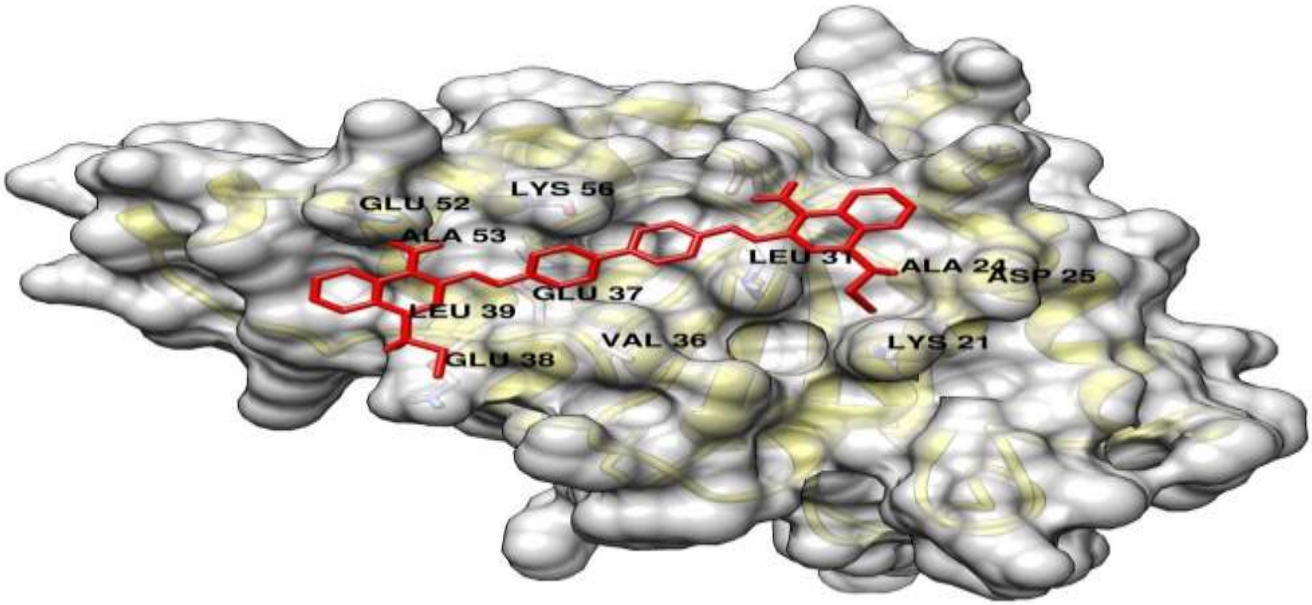


**Figure-9. Protein Docking structure of YB4 with CR**

## Discussion

The textile industry wastewater loaded with dyes, metal ions and salts is among the main causes of water pollution and poses serious threats to environment. This condition even worsens when the metal ions like Cr(VI) are released with different textile and tannery wastewater and mixed with dyes loaded wastewater. There is need to cope this problem through environment friendly and cost-effective approaches. This study was designed to isolate the strain which has the potential to remove the azo dyes and Cr(VI) simultaneously. The results presented that most of the strains showed the removal of RB5 and Cr(VI) more than 50% of initially added their concentrations in 48 h. This diversity in the results might be because of the acclimatization behavior of the strains in the presence of azo dye. This pattern of results has been showed in many other studies (Najme et al., 2015; Imran et al., 2015a; Maqbool et al., 2018; Baig et al., 2019; Hussain et al., 2020). Among these strains, YB4 showed the maximum efficiency for the decolorization of azo dyes and Cr(VI) reduction simultaneously.

Moreover, 16S analysis showed the similarity of the selected strain with the *Alcaligenes* sp. and previous studies showed that *Alcaligenes* sp. has the capability to decolorize the azo dyes (D'Souza et al., 2017; Saha et al., 2017; Ajaz et al., 2019; Mehandia et al., 2020).

It was noted that *Alcaligenes* sp. YB4 possesses the potential to decolorize different structured azo dyes, resulting in varying degrees of decolorization for each type of azo dye. (Hussain et al., 2013; Maqbool et al., 2018; Hussain et al., 2020; Baig et al., 2019). This variation in the functioning of *Alcaligenes* sp. YB4 against the different structured azo dyes can be due to varying adaptation of YB4 for the different structured dyes, or it might be due to variation in bacterium acclimatization with different azo dye (Hussain et al., 2013; Imran et al., 2015b). The decolorization potential could be possible upon the complexity and structure of the dyes, particularly the position of the aromatic ring in the dye and the subsequent interactions of azo bonds (Al-Ansari et al., 2022). In recent years, multifunctioning microbes having ability for dyes decolorization as well as having resistance against the metal ions and salts have been reported in different researches (Anwar et al., 2014; Abbas et al., 2016; Hussain et al., 2020; Baig et al., 2019). While simultaneous removing of dyes and Cr(VI), it was observed that the decolorization rate was initially slow, due to the presence of Cr(VI). However, it later exceeded 80% in the subsequent stages. This decrease in rate of decolorization can be due to toxic effects of Cr(VI) at initial stages for the YB4 (Mahmood et al., 2011; Mahmood et al., 2013; Maqbool et al., 2016). The slow rate of decolorization supported the fact that the strain YB4 initially utilized the Cr(VI) as electron acceptor instead of CR azo dye which has already been discussed (Mahmood et al., 2013; Maqbool et al., 2016).

The maximum CR and Cr(VI) removal was detected at pH 8 in the current study. Numerous studies have shown that pH values ranging from neutral to slightly alkaline conditions offer the maximum potential for decolorizing azo dyes using various strains (Najme et al., 2015; Maqbool et al., 2016; Hussain et al., 2020). Highly acidic and alkaline environments directly affect population growth of the strain and affects the activity of the functional enzymes. Bacterial decolorization is hinder/ed at acidic pH levels by the protonation of azo dyes (Agrawal et al., 2014; Akansha et al., 2022). That's the reason, the acidic and highly alkaline medium decreases the decolorizing capabilities of the strain YB4.

Many other researchers have already reported the maximum decolorization of dyes in media amended with yeast extract (Hussain et al., 2013; Abbas et al., 2016; Maqbool et al., 2018; Hussain et al., 2020; Baig et al., 2019). It might be assumed that the growth of the YB4 might have been enhanced by yeast extract as compared to the other C sources (maltose, D-mannitol, glucose) because it is not only source of C but also N while the others are solely carbon sources (Imran et al., 2016a; Maqbool et al., 2018). With the increase in growth of the strain YB4, it might enhance the production of NADH and NADPH which is key component of azoreductase enzyme, an important enzyme involved in the breaking of azo bonds during the decolorization (Imran et al., 2016b; Maqbool et al., 2018). Increased decolorization due to yeast extract might be associated with riboflavin which is a component of yeast extract and also serves as a redox mediator (Imran et al., 2016a).

The strain *Alcaligenes* sp. YB4 has the capability of removing CR and Cr(VI) even in MSM containing the NaCl salt. Higher concentrations (20, 50, 100 g/L) of NaCl had significantly reduced the simultaneous removal of CR and Cr(VI). Some other

findings also testimony the same results by some other researchers who showed the decolorization potential of various bacteria in the presence of varying NaCl concentrations (Khalid et al., 2008; Hussain et al., 2013; Anwar et al., 2014; Abbas et al., 2016). One of the possible reasons for the decrease in decolorization potential is the inhibitory effect of salt on strain growth. Additionally, plasmolysis can also be a contributing factor to the reduced growth of the strain (Imran et al., 2015b). In addition to influencing growth, salt concentration also has an impact on the activity of enzymes engaged in the decolorization of azo dyes (Moutaouakkil et al., 2003; Dhal et al., 2010; Zilly et al., 2011; Imran et al., 2015b).

This study also revealed the heavy metal's resistance of *Alcaligenes* sp. against different heavy metals ($Pb^{+2}$, $Cd^{+2}$, $Ni^{+3}$, $Co^{+2}$, $Zn^{+2}$, $Cr^{+6}$). The strain YB4 showed variability in resistance potential for different heavy metals. The order for the heavy metal resistance potential was $Cr^{6+}>Co^{2+}>Zn^{2+}>Pb^{2+}>Cd^{2+}>Ni^{2+}$. Several recent studies reported the existence of bacteria that demonstrate resistance to heavy metals in terms of their MIC (Hussain et al., 2013; Mahmood et al., 2017; Hussain et al., 2020; Bilal et al., 2021). Hussain et al. (2020) reported the heavy metal resistance potential of *Pseudomonas* sp. WS-D/183. Their results also showed the varying heavy metal resistance potential of *Pseudomonas* sp. WS-D/183 against various metals. Similarly, Bilal et al. (2022) also reported the heavy metal resistance potential of two rhizospheric *Bacillus* sp. SG36 & SG42. However, the bacterial strain's growth and activity were adversely affected by higher concentrations of heavy metals. Heavy metals are harmful to microbes as they hinder enzymatic functions, serve as redox catalysts in the generation of reactive oxygen species (ROS), interfere with ion regulation, and impact DNA and protein synthesis (Gauthier et al., 2014; Igiri et al., 2018).

The CR and Cr(VI) removing capability of YB4 was depressed as the concentration of heavy metal increases. It might be because the metals can retard microbial activities (Mahmood et al., 2013). Recently, there have been reports of dye decolorizing bacteria that not only exhibit resistance to metals but also demonstrate the ability to decolorize dyes in the presence of metal ions (Maqbool et al., 2016; Hussain et al., 2020; Baig et al., 2019; Louati et al., 2019). As the concentration of heavy metal ions increased, the rate of decolorization of azo dye by *Alcaligenes* sp. YB4 was decreased, because of toxic effect and stress of heavy metals on bacterial growth, enzymatic activities that effect the functioning capability of bacteria (Chen et al., 2003; Mahmood et al., 2011, 2013; Maqbool et al., 2016).

173 amino acids were found after the translation of nucleotide sequence in this study. BLASTp analysis showed the resemblance with the FMN-dependent NADH-azoreductase. Further, approximately 90% of the amino acids fell within the favored region of the Ramachandran plot, thereby confirming the structural stability of the nature. Based on the steric interference of the residues, molecular docking helps determine the dye molecules' binding location (Basharat et al., 2017; Basharat et al., 2021). Various researchers have predicted the protein structure of azo reductase in their studies (Sarkar et al., 2020; Abbas et al., 2020; Bafana, 2022).

Further, ligand protein docking results confirmed the interaction of CR structure on the azoreductase. Numerous investigations have previously documented the docking of azo dyes with azoreductase (Sarkar et al., 2020; Krithika et al., 2021; Mishra et al., 2023). Specifically, Basharat and Yasmin (2022) conducted a prior study in which they predicted the binding of azo dyes to the azoreductase enzyme derived from *Alcaligenes* sp. Their findings revealed the presence of hydrogen and ionic bonds between CR and azoreductase, encompassing both bonded and non-bonded interactions. Additionally, a Pi interaction was identified alongside the hydrogen and ionic bonds. Furthermore, Thakuria et al. (2015) previously reported interactions between azoreductase (AzoR) and CR and Methyl Orange (MO) in *Pseudomonas putida*.

## Conclusion

It is concluded that, *Alcaligenes* sp. strain YB4 have potential to remove the CR and Cr(VI) simultaneously in different conditions and NADH-ubiquinone: oxidoreductase may be responsible for the decolorization of azo dye. Protein-ligand docking study also confirmed the binding of CR dye on the azoreductase from which it can be hypothesized that their interaction may be the key role for the CR degradation. Therefore, the current study emphasizes on application of *Alcaligenes* sp. for bioremediation of textile wastewater on commercial level.

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## Contribution of Authors

Yasir Bilal executed the experiments of this study, data curation and writing the first draft of the article. Sabir Hussain supervised all the experiments and arranged the funding for equipment and chemicals for this study. Tanvir Shahzad and Faisal Mahmood conducted various analyses and proof read the manuscript. Muhammad Shahid helped in the bioinformatic analysis of the amplified azoreductase gene.

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